Lenti-X™ Lentiviral Expression Systems User Manual

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Table of Contents

I. Introduction 3
A. Gene Transfer and Expression Using Recombinant Lentiviruses 3
B. Lenti-X Vectors 3
C. Lenti-X HT Packaging Systems 3

II. Additional Materials Required 6

III. Safety Guidelines for Working with Lentiviruses 8

IV. Plasmid Vector Manipulations 9
A. General Molecular Biology Techniques 9
B. Plasmid Vector Propagation & Construction of Your Customized Lenti-X Vector 9

V. Cell Culture Guidelines 10
A. General Cell Culture and Lentivirus Information 10
B. **Protocol:** Starting Lenti-X 293T Cell Line Cultures from Frozen Stock 10

VI. Producing Lentivirus from Lenti-X Vectors 11
A. **Protocol:** Producing Lentivirus with Lenti-X 293T Cells and Lenti-X HT Packaging Systems 11

VII. Determining Lentiviral Titer 12
A. Introduction 12
B. **Protocol:** Determining Viral Titer Using Antibiotic Selection 12
C. Alternative Titration Methods 13

VIII. Transducing Target Cells with Lenti-X Viruses 14
A. **Protocol:** Transducing Target Cells with Lenti-X Viruses 14

IX. Troubleshooting Guide 15

X. References 17

Appendix A: Additional Protocols 18
A. **Protocol:** Titrating Antibiotics for Selecting Stable Cell Lines 18
B. **Protocol:** Concentrating Virus 18

Appendix B: Additional Viral Infection Methods 19

List of Figures

Figure 1. Clontech has Lenti-X expression systems and vectors for many applications. 4
Figure 2. Lentivirus production with the Lenti-X HT Packaging System and Lenti-X 293T cells. 5
Figure 3. Tetracycline activity in bovine sera. 6
Figure 4. Advanced features of NucleoBond Xtra Maxi and Midi Columns and NucleoBond Finalizer. 9
Figure 5. High infectivity of supernatants produced by the Lenti-X HT Packaging System. 14

List of Tables

Table I. Troubleshooting Guide for Lenti-X Expression Systems 15
Table I. Troubleshooting Guide for Lenti-X Expression Systems (continued) 16
Table II. Recommended Concentrations for Selection Antibiotics (µg/ml) 18

I. Introduction

A. Gene Transfer and Expression Using Recombinant Lentiviruses

Recombinant lentiviral vectors are powerful and efficient tools for transferring heritable genetic material into the genome of virtually any cell type (Ausubel *et al.*, 1995; Coffin *et al.*, 1996). Lentiviruses are perhaps the most versatile of retroviruses since they are able to infect, transduce, and sustain expression in almost any mammalian cell, including dividing and nondividing cells, stem cells, and primary cell cultures. In Lenti-X systems, high titers of recombinant, replication-incompetent virions are easily generated by cotransfecting one of our many Lenti-X expression vectors (containing your gene of interest or "GOI"; Figure 1) along with a **Lenti-X HT Packaging Mix**, into the **Lenti-X 293T Cell Line** (Cat. No. 632180). The Lenti-X HT Packaging Mix and the Lenti-X HT Ecotropic Packaging Mix are optimized mixtures of plasmids that respectively express specific ratios of the viral proteins necessary for high efficiency packaging of lentiviral vector transcripts into infectious, VSV-G- or ecotropically-pseudotyped, lentiviral particles. The VSV-G envelope protein works for almost any cell type, while the ecotropic envelope glycoprotein (gp70) from MLV enables mouse and rat cells to be transduced with high efficiency. The lentiviral supernatants produced by the transfected packaging cells can then used to infect and transduce target cells to express your GOI, fusion protein, or shRNA. Clontech has developed several highly advanced Lenti-X expression systems that provide the broad cellular tropisms of pseudotyped lentivirus; very high titers of safe, nonreplicating virus; and excellent transgene expression levels (*Clontechniques*, October 2007).

B. Lenti-X Vectors

Clontech offers Lenti-X vectors for many applications (Figure 1). These vectors all possess the requisite HIV-1 LTRs and the lentiviral packaging signal (Ψ) as well as other elements that improve transgene expression, viral titer, and overall vector function.

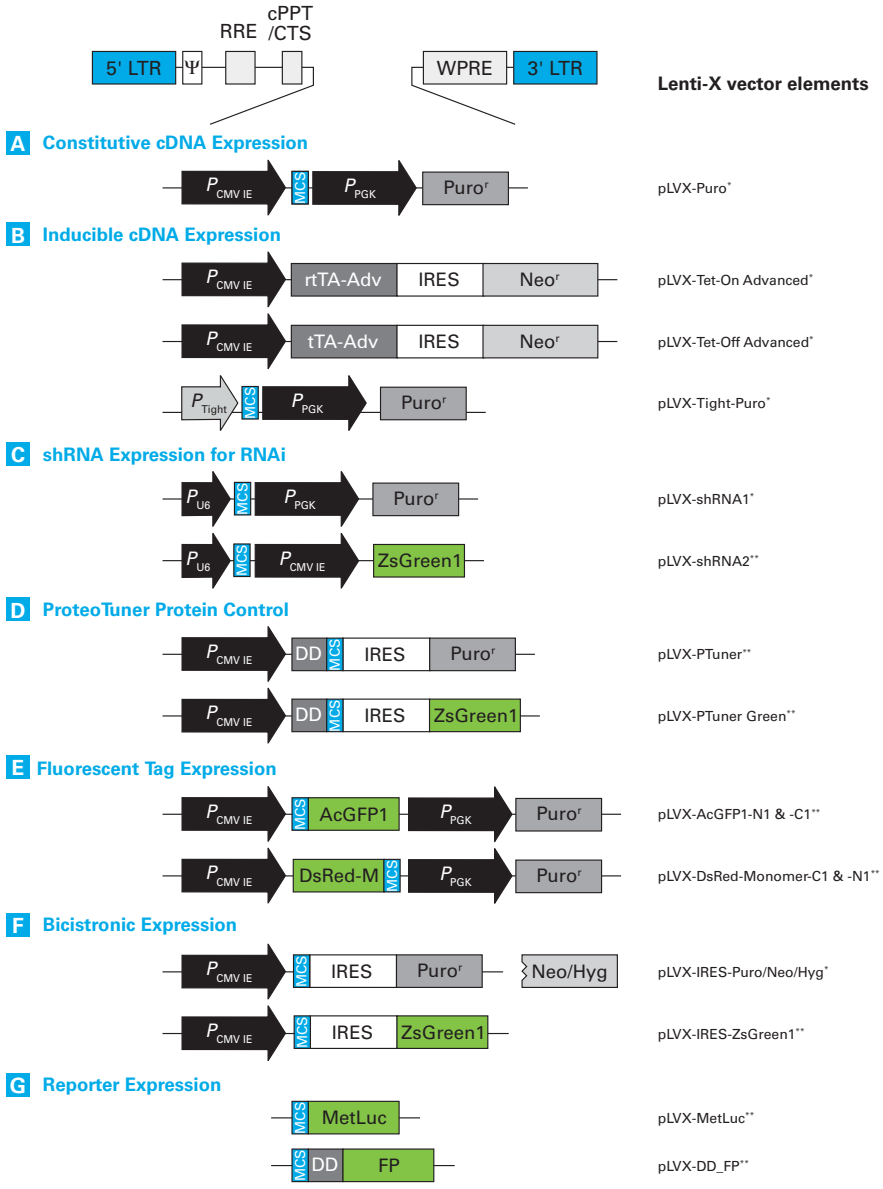
- **WPRE**: A woodchuck hepatitis virus posttranscriptional regulatory element prevents poly A site readthrough, promotes RNA processing and maturation, and increases nuclear export of RNA (Zufferey *et al.* 1999; Higashimoto *et al.,* 2007). It works the context of viral genomic transcripts in packaging cells to enhance vector packaging and increase the viral titers. In addition, the WPRE boosts expression of your GOI in transduced target cells by facilitating the maturation of mRNA transcripts produced by the vector's internal promoter (e.g. P_{CMV} or P_{Tight}).
- **cPPT/CTS**: A central polypurine tract/central termination sequence creates a "DNA flap" that increases nuclear importation of the viral genome during target cell infection. The cPPT/CTS element improves vector integration and transduction efficiency (Zennou *et al.*, 2000).
- **RRE**: A Rev response element helps to increase titers by promoting the nuclear export of unspliced viral genomic RNA (Cochrane, *et al.*, 1990).

C. Lenti-X HT Packaging Systems

To produce recombinant lentivirus for target cell infection, Lenti-X plasmid vectors must be cotransfected into Lenti-X 293T cells, along with a Lenti-X HT Packaging Mix, in order to assemble your vector and accompanying GOI into infectious virions (Figure 2).

- **Highest Titers**: Lenti-X HT Packaging Mixes are plasmid mixtures that provides the necessary viral packaging components in specific, optimized ratios. When your vector and packaging mix are transfected into Lenti-X 293T cells using the **Lentiphos™ HT** transfection reagents, the packaging mix expresses the Pol (RT & IN), Tat, Rev, and Gag lentiviral proteins, and either the VSV-G envelope protein or the ecotropic gp70 envelope protein from MLV (Wu *et al.*, 2000; *Clontechniques,* October 2007). The recombinant viral vector is then replicated and assembled into complete, pseudotyped virus particles (Figure 2). The packaging mix includes an expression vector for the Tet-Off® transcriptional activator (tTA) and uses Tet transactivation to produce very high expression levels of specific viral proteins (Gossen & Bujard, 1992). This optimized expression strategy, combined with high-efficiency transfection, produces very high virus titers that can be as much as 25–50 times higher than other commercially available systems. Lenti-X supernatants can very often be used to infect target cells directly without prior concentration.

I. Introduction continued



* Vectors available as part of an expression system.
** Vectors available separately.

Figure 1. Clontech has Lenti-X expression systems and vectors for many applications. Lenti-X vectors contain sequence elements that facilitate lentiviral packaging and/or boost transgene expression. These include the HIV-1-based LTRs, packaging signal (Ψ), Rev response element (RRE), and central polypurine tract/central termination sequence (cPPT/CTS), as well as a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). All vectors are designed to be used with a Lenti-X HT Packaging System and the Lenti-X 293T Cell Line, which together produce very high titers of pseudotyped lentivirus for transducing virtually any cell type. **Panel A:** The pLVX-Puro Vector of the Lenti-X Expression System is for constitutive expression of a GOI. **Panel B:** The regulator and response vectors of the Lenti-X Tet-On and Tet-Off Advanced Inducible Expression Systems are used to control GOI expression with doxycycline. **Panel C:** The vectors of the Lenti-X shRNA Expression System are for shRNA-mediated inhibition of specific genes through RNAi. The vectors contain either a puromycin resistance or a fluorescent marker gene. **Panel D:** The vectors of the Lenti-X ProteoTuner Systems are used to create and express a highly labile version of your protein (containing a degradation domain, or DD), the stability of which is controlled with the Shield1 ligand. N- and C-terminal versions are available. **Panel E:** Lenti-X fluorescent tag vectors can be used to express your protein as a C- or N-terminal fusion protein containing a fluorescent marker. **Panel F:** Lenti-X bicistronic expression vectors allow your protein and either a selectable marker or fluorescent protein to be coexpressed from a single mRNA transcript. **Panel G:** Lenti-X reporter vectors allow you to monitor promoter activation with a chemiluminescent or fluorescent reporter. DD: degradation domain; IRES: internal ribosome entry sequence; MCS: multiple cloning site; MetLuc: *Metridia* luciferase; $P_{CMV\ IE}$: cytomegalovirus immediate early promoter/enhancer; P_{PGK}: phosphoglucokinase promoter; P_{Tight}: the modified Tet-responsive promoter; P_{U6}: human U6 snRNA promoter (RNA Pol III).

I. Introduction continued

C. Lenti-X HT Packaging Systems (cont'd)

- **Highest Safety:** For added biosafety, the genes that express the viral packaging proteins have been split onto different plasmids to prevent the collective inclusion of these coding sequences into viral particles during the packaging process. The lack of sequence homology between the packaging mix plasmids and our Lenti-X Vectors also prevents transfer via homologous recombination. This split-gene, *trans*-expression strategy effectively prevents the production of replication-competent lentivirus, e.g. the viruses cannot replicate autonomously in target cells.

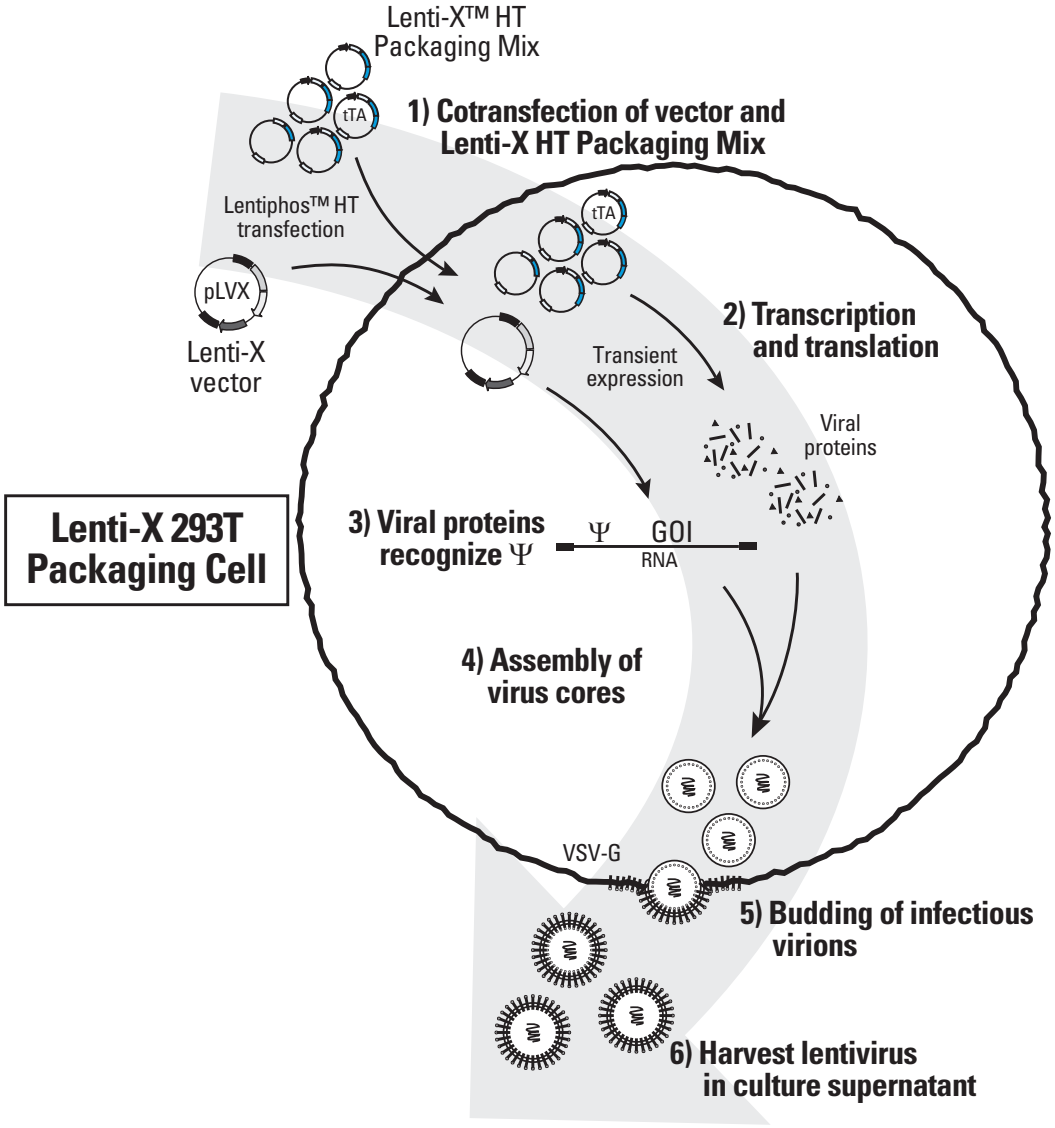


Figure 2. Lentivirus production with the Lenti-X HT Packaging System and Lenti-X 293T cells. Initially, cotransfection of a Lenti-X vector and the Lenti-X HT Packaging Mix (Step 1) results in the production of the corresponding recombinant lentiviral genomic RNA transcript and viral packaging proteins (Step 2). A vector in the packaging mix encodes the Tet-Off transactivator (tTA), which produces extra-high expression of specific packaging proteins via Tet-Off transactivation. Recognition of the packaging sequence (Ψ) on the recombinant viral RNA genome by the packaging proteins (Step 3) results in the assembly of viral cores, which are transported to the cell membrane (Step 4). Cores are then enveloped by cellular membrane containing aggregated VSV-G or ecotropic/gp70 envelope proteins. Mature, infectious virions then bud from the cell (Step 5) and are collected in the medium (Step 6). While the virions are infectious, they lack several critical genes required for their subsequent replication and production in target cells. The use of multiple plasmids with which to express the viral proteins adds a strong measure of safety to virus production since several low-frequency recombination events would need to occur in order to regenerate a replication-competent viral genome.

II. Additional Materials Required

A. Cell Lines for Lentivirus Packaging and Titration

- **Lenti-X 293T Cell Line:** An HEK 293T-derived cell line optimized for virus production. To obtain high-titer supernatants of infectious lentivirus, your Lenti-X vector and a **Lenti-X HT Packaging Mix** are cotransfected into Lenti-X 293T cells using the **Lentiphos™ HT** transfection reagents. The transfected cells will consistently produce very high titers of pseudotyped lentivirus. Alternatively, the HEK 293T/17 cell line from American Type Culture Collection (ATCC No. CRL-11268™) can be used, but is not as optimized for Lenti-X Systems.
- **HT-1080 cell line:** American Type Culture Collection HT-1080 (ATCC No. CCL-121™) [Recommended]. This cell line is easily transduced by recombinant lentiviruses and is frequently used for lentiviral titration. Alternatively, virus stocks can be titrated with the **Lenti-X qRT-PCR Titration Kit** (Cat. No. 632165)

B. Mammalian Cell Culture Supplies

- **Lenti-X 293T Cell Line growth medium:** 90% Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and 3.7 g/L sodium bicarbonate (Sigma-Aldrich Co., No. D5796); and 10% *tetracycline-free* fetal bovine serum. *Add 1 mM sodium pyruvate.*
- **HT-1080 growth medium:** 90% Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and 3.7 g/L sodium bicarbonate (Sigma-Aldrich Co., No. D5796); and 10% fetal bovine serum. *Add 1 mM sodium pyruvate.*
- **Tetracycline-free fetal bovine serum** (FBS; see important information below). We *strongly recommend* using **Tet System Approved FBS** (Cat. Nos. 631101 & 631106) for all packaging cell transfections and for culturing target cells when using a Lenti-X Tet-Advanced Inducible Expression System.
- Cell growth medium and supplies specific for your target cells

Tetracycline-Free Fetal Bovine Serum (FBS) for Packaging Cell and Target Cell Culture

Many lots of bovine sera are contaminated with tetracycline (Tc) or its derivatives which can affect basal expression or inducibility in Tet Expression Systems (Figure 3). It is critical that the FBS used for cell culture not interfere with Tet-responsive expression.

- The Lenti-X HT Packaging Mix utilizes Tet-Off transactivation to drive high-level expression of specific viral packaging proteins. The presence of Tc contaminants in FBS will reduce expression of these important components and will negatively affect viral titers. *Therefore, 293T cells that host the Lenti-X HT Packaging System must be cultured in medium containing Tc-free FBS.*
- Tc-contaminants in FBS will also significantly diminish the performance of the Tet-On and Tet-Off Advanced Systems in target cells.
- These problems can be eliminated by using a **Tet System Approved FBS** (Cat. Nos. 631101 & 631106) from Clontech. These sera have been functionally tested in our Tet Systems and found to be free of contaminating Tc activity.

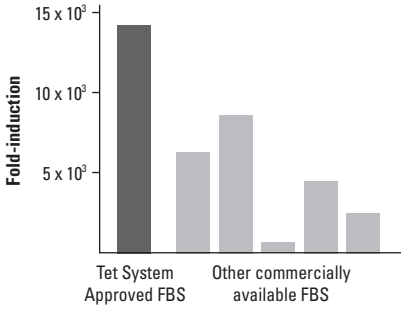


Figure 3. Tetracycline activity in bovine sera. The CHO-AA8-Luc Tet-Off Control Cell Line was grown in media prepared with different lots of FBS. Average uninduced expression level = 0.21 RLU (n=21, S.D.=0.07); maximum expression levels varied from 123 to 3,176 RLU.

II. Additional Materials Required continued

B. Mammalian Cell Culture Supplies (continued)

- Sodium pyruvate solution, 100 mM, sterile filtered (Sigma-Aldrich Co., No. S8636), for supplementing cell culture media.
- Penicillin/streptomycin solution of 10,000 units/ml penicillin G sodium and 10,000 µg/ml streptomycin sulfate (100X; Sigma-Aldrich Co., No. P0781)
- Trypsin-EDTA (Trypsin; Sigma-Aldrich Co., No. T3924)
- Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich Co., No. D8662)
- L-glutamine solution, 200 mM, sterile filtered (Sigma-Aldrich Co., No. G7513) [Optional]
- Cell Freezing Medium, with or without DMSO (Sigma-Aldrich Co., No. C6164 or No. C6039)
- Tissue culture plates (100 mm) for packaging cell transfections; other plates and flasks as required
- Polystyrene culture tubes, 12 x 75 mm (e.g., BD Falcon™ No. 352054), for packaging cell transfections.
- Sterile microfuge tubes (1.5 ml) for use in titrating virus stocks; and cryovials for freezing virus stocks.
- Crystal violet (Sigma-Aldrich Co., No. C3886), 1% solution prepared in ethanol, for staining colonies of transduced cells in the virus titration protocol (Section VII.B).
- Cloning cylinders (PGC Scientific, No. CORN31666, -31668, or -316610), for isolating clones of stable transductants.

C. Lenti-X qRT-PCR Titration Kit

For accurate and consistent transductions, we highly recommend titrating your lentiviral stocks. The **Lenti-X qRT-PCR Titration Kit** (Cat. No. 632165) provides a fast and simple qRT-PCR-based titration method (*Clontechniques,* January 2008). The kit determines viral RNA genome content using qRT-PCR and SYBR® technologies, and titrates virus stocks in ~4 hr.

D. Antibiotics for Selecting Transduced Cells

G418 (Cat. No. 631307), **Puromycin** (Cat. Nos. 631305 & 631306), **Hygromycin B** (Cat. No. 631309) are used for performing drug selection of target cells transduced with Lenti-X viruses having the respective resistance genes, and for titrating the corresponding Lenti-X virus stocks by drug selection. Prior to using these antibiotics, determine the optimal selection concentration for each cell type as described in Appendix A.

E. Polybrene for Viral Transductions

Polybrene (hexadimethrine bromide; Sigma-Aldrich, No. H9268) is needed for the standard infection/transduction protocol to facilitate lentiviral gene transfer. Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimal polybrene concentration for your target cells (maximal infectivity with minimal toxicity) should be determined empirically by testing concentrations in the range of 2–12 µg/ml. For cells that are especially sensitive to polybrene, consider using RetroNectin® Reagent.

F. RetroNectin® Reagent for Enhanced Viral Transductions

RetroNectin Reagent (available from Clontech Laboratories, Inc. or Takara Bio USA, Cat. Nos. TAK 100A, TAK 100B) is a recombinant fragment of fibronectin (CH-296) that can be used to greatly improve retroviral and lentiviral transduction efficiencies (*Clontechniques*, October 2008). RetroNectin is coated onto tissue culture plates to provide a substratum that binds both viruses and cells. The colocalization of virus and cells on this novel substratum improves cell-virus contact and enhances transduction. This is especially useful for cells grown in suspension (e.g. lymphocytes and lymphocyte cell lines) and other cells that are difficult to transduce, such as hematopoietic stem cells; or for cells that may be especially sensitive to polybrene. Visit **www.takarabiousa.com** for more information.

III. Safety Guidelines for Working with Lentiviruses

The protocols in this User Manual require the production, handling, and storage of infectious lentivirus. It is imperative to fully understand the potential hazards of, and necessary precautions for, the laboratory use of lentiviruses.

The National Institute of Health and Center for Disease Control have designated recombinant lentiviruses as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. The pseudotyped lentiviruses packaged from the HIV-1-based vectors described here are capable of infecting human cells. The viral supernatants produced by these lentiviral systems could, depending on your insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*.

For these reasons, due caution must be exercised in the production and handling of any recombinant lentivirus. **The user is strongly advised not to create pseudotyped lentiviruses capable of expressing known oncogenes.**

For more information on Biosafety Level 2 agents and practices, download the following reference:

- *Biosafety in Microbiological and Biomedical Laboratories (BMBL),* Fifth Edition (February 2007) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH. Available on the web at **http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**

Biosafety Level 2: The following information is a brief description of Biosafety Level 2. *It is neither detailed nor complete.* Details of the practices, safety equipment, and facilities that combine to produce a Biosafety Level 2 are available in the above publication. If possible, observe and learn the practices described below from someone who has experience working with lentiviruses.

Summary of Biosafety Level 2:

- **Practices:**
  - Standard microbiological practices
  - Limited access to work area
  - Biohazard warning signs posted
  - Minimize production of aerosols
  - Decontaminate potentially infectious wastes before disposal
  - Use precautions with sharps (e.g., syringes, blades)
  - Biosafety manual defining any needed waste decontamination or medical surveillance policies
- **Safety equipment:**
  - Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is unrecirculated
  - PPE: protective laboratory coats, gloves, face protection as needed
- **Facilities:**
  - Autoclave available for waste decontamination
  - Chemical disinfectants available for spills

IV. Plasmid Vector Manipulations

A. General Molecular Biology Techniques

These protocols contain only general information for propagating plasmid vectors and for preparing your customized expression construct in a Lenti-X Vector. For users requiring more information on standard molecular biology practices and cloning techniques, we recommend the following laboratory references:

- *Current Protocols in Molecular Biology* ed. by F. M. Ausubel *et al.* (1995, John Wiley & Sons, NY).
- *Molecular Cloning: A Laboratory Manual* ed. by J. Sambrook *et al.* (2001, Cold Spring Harbor Laboratory Press, NY).

B. Plasmid Vector Propagation & Construction of Your Customized Lenti-X Vector

1. To ensure that you have a renewable source of plasmid DNA, transform each of the plasmid vectors provided in this kit into a *E. coli* host strain suitable for viral vectors, such as **Supercharge EZ10 Electrocompetent Cells** (Cat. No. 636756). Consult the Vector Information Packet provided with each Lenti-X vector for further DNA propagation details.
2. To purify plasmid DNA for cloning purposes, use a suitable **NucleoBond® or NucleoSpin® Kit.** See **www.clontech.com** for available kits and options.
3. Using standard cloning techniques, insert your coding sequence into the vector's multiple cloning site (MCS). Consult the Vector Information Packet provided with each Lenti-X vector for additional cloning details. You can also use an **In-Fusion™ 2.0 PCR Cloning Kit** (Cat. No. 639607) which allows PCR products to be easily cloned into any linearized vector.

Note: Depending on the Lenti-X vector selected, your GOI sequence (cDNA or gene fragment) may require an ATG initiation codon. In such cases, addition of a Kozak consensus ribosome binding site (Kozak, 1987) may improve expression levels, but this is generally not required. ***However, the fragment or cDNA must not contain a polyadenylation signal.*** The insertion of such sequences between viral LTRs can cause premature cleavage and polyadenylation during transcription of the viral genome. This interferes with the production of viable recombinant virions (Coffin et. al, 1997).

4. Perform a midi- or maxi-scale plasmid DNA preparation for each plasmid that will be transfected into the packaging cells. For guaranteed transfection-grade plasmid DNA, we recommend using **NucleoBond Xtra Midi Plus** or **Maxi Plus Kits** (Figure 4; Cat. Nos. 740412.10 and 740416.10). For rapid production of *endotoxin-free*, transfection-grade plasmid DNA, use **NucleoBond Xtra Midi EF Plus** or **Maxi EF Plus Kits** (Cat. Nos. 740422.10 and 740426.10).

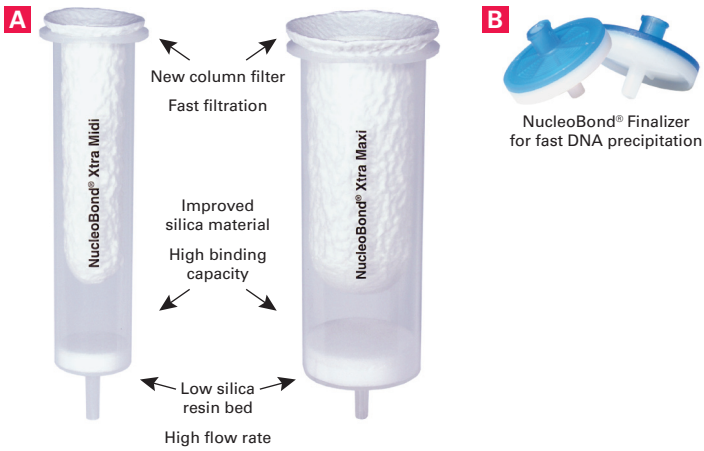


Figure 4. Advanced features of NucleoBond Xtra Maxi and Midi Columns and NucleoBond Finalizer. NucleoBond Xtra columns contain a high-flow column filter that minimizes clogging and clears debris from cell lysates during column loading. An improved silica resin provides high DNA-binding capacity, and a wide column diameter keeps the resin bed low for maximum flow rates (**Panel A**). The NucleoBond Finalizer system speeds preparation and increases purity by capturing precipitated DNA on a syringe filter where it can be easily washed and eluted (**Panel B**).

V. Cell Culture Guidelines

A. General Cell Culture and Lentivirus Information

The protocols in this User Manual provide only general guidelines for lentivirus use and mammalian cell culture techniques. Perform all steps involving cell culture using sterile technique in a Biosafety Level 2 tissue culture hood that has been approved for use with lentiviruses. For users requiring more information on lentiviruses, retroviruses, and mammalian cell culture, we recommend the following general references:

- *Retroviruses,* ed. by J. M. Coffin, S. H. Hughes & H. E. Varmus (1997, Cold Spring Harbor Laboratory Press, NY)
- *Culture of Animal Cells,* 5th Edition, ed. by R. I. Freshney (2005, Wiley-Liss, NY)
- *Current Protocols in Molecular Biology,* ed. by F. M. Ausubel, *et al.* (1995, Wiley & Sons)

B. Protocol: Starting Lenti-X 293T Cell Line Cultures from Frozen Stock

Protocol
1 hr

Frozen cells should be cultured immediately upon receipt, or as soon as possible thereafter. If culturing is significantly delayed after receipt, decreased cell viability may result. For HEK 293-based cell lines, we recommend using collagen-coated plates or flasks for efficient culturing of frozen stocks. Vessels coated with compounds other than collagen may also provide suitable growth substrates (e.g. poly-L-lysine), but only collagen has been tested at Clontech. Once recovered, the cells may be cultured directly on tissue culture plastic. However, if adherence is poor, we recommend using only collagen-coated vessels.

To prevent osmotic shock and maximize cell survival, perform the following:

1. Warm ~25 ml of complete culture medium in a 37°C water bath. See Section II.B for medium composition.

   Note: Be sure to use Tet System Approved Fetal Bovine Serum (Cat. Nos. 631101 & 631106) when using these cells with the Lenti-X HT Packaging System (Cat. 632160).

2. Thaw the vial of cells rapidly in a 37°C water bath with gentle agitation. Immediately upon thawing, wipe the outside of the vial with 70% ethanol. All of the operations from this point on should be carried out in a laminar flow tissue culture hood under strict aseptic conditions. Unscrew the top of the vial slowly and, using a pipet, transfer the contents of the vial to a 15 ml conical centrifuge tube containing 1 ml of pre-warmed medium. Mix gently.
3. Slowly add an additional 4 ml of fresh, pre-warmed medium to the tube and mix gently.
4. Add an additional 5 ml of pre-warmed medium to the tube, mix gently. Centrifuge at 100 x g for 5 min, carefully aspirate the supernatant, and GENTLY resuspend the cells in complete medium. (This method removes the cryopreservative and can be beneficial when resuspending in small volumes. However, be sure to treat the cells gently to prevent damaging fragile cell membranes.)
5. Mix the cell suspension thoroughly and add to a suitable culture vessel. Gently rock or swirl the dish/flask to distribute the cells evenly over the growth surface and place it in a 37°C humidified incubator (5–10% CO_2 as appropriate) for 24 hr.
6. The next day, examine the cells under a microscope. If the cells are well-attached and confluent, they can be passaged for use. If the majority of cells are not well-attached, continue culturing for another 24 hr. Complete attachment of newly thawed cultures of HEK 293-based cell lines, may require up to 48 hr.
7. Once the culture has been started and the cells are growing normally, you should prepare frozen aliquots to provide a renewable source of cells. Consult the Lenti-X 293T Cell Line Protocol-at-a-Glance (PT4058-2) for a cell freezing protocol.

VI. Producing Lentivirus from Lenti-X Vectors

Protocol 2–4 days

A. Protocol: Producing Lentivirus with Lenti-X 293T Cells and Lenti-X HT Packaging Systems

To obtain the highest titers from the Lenti-X HT Packaging System, use the Lenti-X 293T Cell Line and adhere strictly to the following protocol, especially with respect to: **(1) culture size and volume; (2) DNA amounts and transfection-grade quality; (3) tetracycline-free serum in Lenti-X 293T growth media; and (4) incubation times**.

All Lentiphos HT transfection reagents, volumes, and conditions are optimized for use with Lenti-X Vectors, the Lenti-X HT Packaging Mix, and Lenti-X 293T cells. Use 100 mm tissue culture plates and be sure to use **Tet System Approved FBS** (guaranteed Tc-free), both in the transfection medium (Step 1) and in the medium used to collect the virus (Step 8). Tetracycline-contaminated serum is detrimental to the expression of essential packaging components in the Lenti-X Packaging System (see Section II.B).

Attention

Perform all steps in a sterile tissue culture hood. Lentivirus requires the use of a Biosafety Level 2 facility. Recombinant pseudotyped lentiviruses packaged from HIV-1-based vectors are capable of infecting human cells. Know and use appropriate safety precautions (See Section III).

STOP!
Don't forget:
- Tet System Approved FBS
- 100 mm culture plates
- Transfection-grade DNA

1. One day before the transfection, plate sufficient Lenti-X 293T cells to achieve 50–80% confluency on the day of the transfection. Generally, we plate 4–5 x 10^6 cells/100 mm plate in 10 ml of complete growth medium containing Tc-free FBS. Keep the cells in the incubator until just before you are ready to add the transfection mixture (Step 6).
2. In a 12 x 75 mm polystyrene culture tube (e.g., BD Falcon™ No. 352054), add **Lenti-X HT Packaging Mix** (15 µl), your **Lenti-X Vector DNA** (3 µg) , and sufficient **Sterile H_2O** to achieve a final volume of 438 µl. For example:

15 µl	Lenti-X HT Packaging Mix
6 µl	pLVX-Puro plasmid DNA, (e.g. 3 µg at 0.5 µg/µl)
417 µl	Sterile H_2O
438 µl	**Total Volume**

3. Add 62 µl of **Lentiphos1** solution to the DNA solution and vortex thoroughly.
4. While vortexing the DNA/Lentiphos1 solution, add 500 µl of **Lentiphos2,** dropwise into the tube.
5. Incubate at room temperature for 5–10 min to allow the DNA precipitate to form.
6. Remove the plate(s) of Lenti-X 293T cells from the incubator. Gently vortex the transfection solution, and add the entire contents of the tube (1 ml), dropwise, to the cell culture medium.
7. Gently move the plate(s) back and forth to distribute the transfection solution evenly. Incubate the plate(s) at 37°C for 8 hr to overnight in a CO_2 incubator. Do not disturb the plates once they are placed in the incubator.
8. After 8 hr to overnight, replace the transfection medium with 10 ml fresh complete growth medium (containing Tc-free FBS) and incubate at 37°C for 24–48 hr. Viral titers will generally be highest at 48 hr after the start of transfection. *Caution: discarded medium contains live lentivirus.*
9. Harvest the lentivirus-containing supernatants. *Caution: supernatants contain live lentivirus.* Pool similar stocks, if desired. Centrifuge briefly (500 x g for 10 min) or filter through a 0.45 µm filter.

   Note

   Note: If filtering, use only cellulose acetate or polyethersulfone (PES) (low protein binding) filters. Do not use nitrocellulose filters. Nitrocellulose binds surface proteins on the lentiviral envelope and destroys the virus.
10. Either titrate the virus stock (Section VII), use the virus to transduce target cells, or freeze the stock in aliquots as described in Step 11.
11. To store the virus stock and avoid multiple freeze-thaw cycles, aliquot the cleared supernatant into single-use cryovials. Store tubes at –80°C. No cryoprotectant is required.

    Note: Titers can drop as much as 2–4 fold with each freeze-thaw cycle (Higashikawa & Chang, 2001; Kwon *et al.*, 2003).

VII. Determining Lentiviral Titer

A. Introduction

To produce consistent transduction results using a known multiplicity of infection (MOI), it is necessary to titrate your Lenti-X virus stocks. Freshly harvested virus stocks can be titrated immediately, or frozen in aliquots at –80°C and then titrated. Note that each freeze-thaw cycle can reduce the functional titer of the virus stock by up to 2–4 fold. Titer values will depend heavily on the cell type and method used for titration. In addition, there may be significant differences between titers determined in cells typically used for titration (e.g. HT-1080) and the number of target cells that are ultimately transduced. However, titrations are important for determining the relative virus content of stocks prepared from different vectors, and for:

- Confirming the viability of virus stocks.
- Determining the optimal transduction conditions
- Adjusting the MOI to control the viral copy number of transduced cells
- Determining the maximum number of cells that can be infected by a virus stock.

Titration can be accomplished using different methods, depending on the presence of a marker and its type:

Lenti-X qRT-PCR titration can be used with any lentiviral vector.

- **qRT-PCR**. Clontech offers a convenient **Lenti-X qRT-PCR Titration Kit** (Cat. No. 632165), which rapidly determines the viral content of supernatants, by employing One-Step qRT-PCR and SYBR® Green chemistry in a 4 hr protocol. This method can be used with any lentiviral vector, regardless of the marker involved, and is beneficial for comparing the titers of different vectors and for titrating freshly harvested virus stocks.
- **Flow cytometry.** For Lenti-X vectors containing a fluorescent marker, cells can be transduced using the protocol in Section B, followed by counting the cells ~24 hr later using fluorescence and flow cytometry. Titers determined in this manner are generally higher than those determined by antibiotic selection.
- **Antibiotic selection.** For Lenti-X vectors that contain a selectable marker, cells are infected with serial dilutions of the virus stock and then selected for stable transductants using the appropriate antibiotic. Titers are calculated from the number of drug-resistant colonies that develop after selection is completed.

B. Protocol: Determining Viral Titer Using Antibiotic Selection

Protocol 7–14 days

1. Plate HT-1080 cells (or another cell line) in one 6-well plate the day before performing the titration infections. Plate 2 x 10^5 cells/well, in 2 ml of medium. Reserve at least one well for a "no infection" control.
2. Prepare 20 ml of complete medium and add 60 µl of 4 mg/ml polybrene. This concentration of polybrene (12 µg/ml) will be eventually diluted 3-fold for a final concentration of 4 µg/ml during transduction.

   Note: Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimum final concentration of polybrene may be determined empirically but generally falls within a range of 2–12 µg/ml. Excessive exposure to polybrene (>24 hr) can be toxic to cells.

3. Prepare cleared viral supernatant from the transfected Lenti-X 293T packaging cells (Section VI). This is your virus stock.
4. Prepare six, 10-fold serial dilutions of the virus stock as follows:
   a. Add 1.35 ml of medium containing polybrene (from Step 2) to each of six sterile and numbered 1.5 ml microfuge tubes.
   b. Add 150 µl of the virus stock (from Step 3) to tube 1. Mix gently.
   c. Transfer 150 µl from tube 1 to tube 2 and mix. Continue making serial dilutions by transferring 150 µl from each successive dilution into the next prepared tube.
5. Infect the HT-1080 cells by adding 1 ml from each of the 5 least concentrated viral dilutions (Step 4) to the appropriately labeled wells. The final polybrene concentration will be 4 µg/ml in ~3 ml. Centrifuge the cultures to improve transduction efficiency*.

VII. Determining Lentiviral Titer continued

B. Protocol: Determining Viral Titer Using Antibiotic Selection (continued)

6. After infecting for 8–24 hours, remove the supernatants and begin antibiotic selection using the concentration of antibiotic that is optimal for your cell line (Appendix A). *Caution: discarded medium contains live lentivirus.*
7. Allow drug-resistant colonies to form for 7–14 days. Stain the colonies with 1% crystal violet solution (in 10% ethanol), and count.
8. The titer of the virus stock corresponds to the number of colonies generated by the least concentrated dilution, multiplied by the dilution factor. For example, the presence of 4 colonies in the 10^6 dilution would represent a titer of 4 x 10^6 colony forming units.

***Culture Centrifugation Increases Transduction Efficiency**

Centrifuging the plate at 1,200 x g for 60–90 min at 32°C can significantly increase infection efficiency. A room temperature centrifuge is acceptable if a 32°C unit is not available.

C. Alternative Titration Methods

- The Lenti-X qRT-PCR Titration Kit directly quantitates the viral genomes in your virus stock, which is much faster and often more useful than antibiotic selection. Since it does not rely on antibiotic selection, all particles, regardless of genome sequence or infectivity, can be quantitated. Functional titers do not yield accurate measures of virion concentration because infection and transduction efficiencies depend on the cell line being used for titration.
- You may also determine viral titer by infecting HT-1080 cells with serially diluted viral supernatants produced using a control vector containing an easily detectable reporter gene (e.g. LacZ, luciferase, or a fluorescent protein). Test virus infection on both HT-1080 cells and your target cells. Infecting your target cell line will give you a rough, but rapid, estimation of infection success. You can use other cell lines to determine viral titer, but HT-1080 cells are widely accepted as the standard target cell for titrating lentivirus because these cells are transduced very efficiently. Note that the same virus preparation can yield different "apparent" titers on different cells lines due to host cell factors that can produce very different transduction efficiencies.
- Some variations of the drug-resistance colony assay employ either a shorter selection period (3 days; Byun *et al.*, 1996); recently-infected target cells (Tafuro *et al.*, 1996; Miyao *et al.*, 1995); or *in situ* PCR (PRINS; Claudio *et al.*, 2001), but achieve similar results.
- Other methods for the direct quantitation of virus particles include slot blots (Nelson *et al.*, 1998; Murdoch, *et al.*, 1997; Onodera *et al.*, 1997) and PCR applied to viral supernatants (Quinn & Trevor, 1997; Morgan *et al.*, 1990). Reverse transcriptase activity has also been used for titration (Goff *et al.*, 1981).

VIII. Transducing Target Cells with Lenti-X Viruses

Protocol 2–3 days

A. Protocol: Transducing Target Cells with Lenti-X Viruses

The following protocol is a general method for transducing adherent cell lines, such as HT-1080 or HeLa, using polybrene. Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimum final concentration of polybrene may be determined empirically but generally falls within a range of 2–12 µg/ml. However, excessive exposure to polybrene (>24 hr) can be toxic to cells. This protocol can be used as a starting point for determining the optimal transduction conditions for your target cells. Refer to Appendix B for additional references and alternative infection methods. For cells that are difficult to transduce or that might be sensitive to polybrene, **RetroNectin® Reagent** (Takara Bio USA, Cat. Nos TAK 100A & 100B) can be used to greatly improve transduction efficiency.

1. Plate target cells in their complete growth medium, 12–18 hr before transduction.
2. Thaw aliquots of your cleared and titrated lentiviral stock, or use cleared virus stock freshly prepared from packaging cells (Section VI). Mix gently, but do not vortex. Note that each freeze-thaw cycle will decrease titer by ~2–4-fold.
3. Adjust the volume of medium in the target cell cultures to accommodate the addition of virus and polybrene. Use sufficient polybrene to obtain the desired final concentration during the transduction step (e.g. 4 µg/ml).
4. Dilute the lentiviral stock with medium to obtain the desired MOI. If titer values are unknown, use serial dilutions of the virus stock or supernatant such that the total volume of virus represents no more than 1/3 the final volume of culture medium used for transduction. See Information Box below.
5. Add viral supernatant to the cells and transduce for 8–24 hr. Centrifuge the cultures to improve infection efficiency (see Section VII.B). If you are concerned that exposure to either the polybrene or to the viral supernatant (which contains medium conditioned by the packaging cells) may adversely affect your target cells, limit the transduction to 6–8 hr.
6. Remove and discard the virus-containing transduction medium and replace it with fresh growth medium. *Caution: discarded medium contains live lentivirus.*
7. Continue to incubate the cells for 24–48 hr to allow your gene product to accumulate in the target cells.
8. Harvest the cells for analysis or proceed with selection using the appropriate antibiotic.

   Note: To determine the efficiency of transduction, you can subject a small subpopulation of cells to antibiotic treatment and harvest the remaining cells for analysis. The cells should be used as soon as possible, but not earlier than 24 hr after transduction.

Attention

Using Untitrated Lenti-X Virus Stocks and Supernatants

The Lenti-X HT Packaging System is capable of producing very high virus titers. Using excessive amounts of virus can be detrimental to target cell performance and viability. If you have not determined the titer of your virus stock, perform transduction experiments using several different fold-dilutions to test a range of MOIs. At Clontech, our scientists can often transduce an entire 100 mm dish of target cells using 10–100 µl of unconcentrated Lenti-X supernatant (Figure 5).

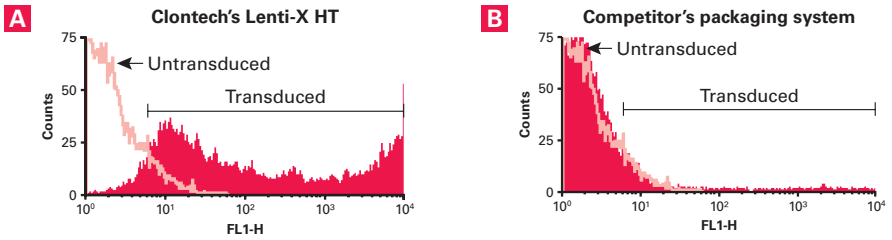


Figure 5. High infectivity of supernatants produced by the Lenti-X HT Packaging System. The Lenti-X HT Packaging System (**Panel A**) and a packaging system from a competitor (**Panel B**) were each used to generate viral supernatants from their respective lentiviral system vector that was engineered to express the ZsGreen1 fluorescent protein. As little as 10 µl of supernatant from the Lenti-X HT Packaging System transduced the majority of these HeLa cells, whereas 10 µl of supernatant from the other system transduced only a small percentage of the cells. Transduced cells were quantitated by flow cytometry.

IX. Troubleshooting Guide

Table I. Troubleshooting Guide for Lenti-X Expression Systems

Description of Problem	Explanation	Solution
A. Vector Cloning		
1. Plasmid is difficult to grow or clone	Some viral vectors may undergo rearrangement between the 5′ and 3′ LTRs when propagated in less-than-optimal *E. coli* host strains	Use **Supercharge EZ10 Electrocompetent Cells** (Cat. No. 636756) to produce high DNA yields and to minimize the potential for DNA rearrangements.
B. Lenti-X 293T Packaging Cells		
1. Poor viability upon thawing	Improper thawing techniques	Use thawing procedure in Section V.B, and/or consult the Lenti-X 293T Cell Line Protocol-at-a-Glance (PT4058-2)
	Incorrect culture medium	Use DMEM with additives listed in Section II.B. Use 10% Tet System Approved FBS (Tc-free).
	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
2. Slow growth	Incorrect culture medium	Use DMEM with additives listed in Section II.B. Use 10% Tet System Approved FBS (Tc-free).
3. Cells do not attach to plate	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
4. Cells appear morphologically different	Passage of cell culture is too high (old cells)	Thaw/purchase new aliquot of Lenti-X 293T cells.
C. Virus Production		
1. Poor transfection efficiency (as determined by GOI or marker expression in the Lenti-X 293T cell line)	Cells plated too densely	Plate 4–5 x 10^6 cells/100 mm plate, or fewer if the cells divide rapidly. Use at 50–80% confluency. See Section VI.
	Transfection is toxic to cells	Use the optimized conditions provided in Section VI.
	Cells harvested or analyzed too soon after transfection	Wait 48 hr after transfection for maximal expression of GOI or marker to determine efficiency.
2. Low titers (<10^5 cfu/ml)	Serum in medium contains tetracycline contaminants	Use Tet System Approved FBS (Cat. No. 631101 & 631106) in the 293T culture medium.
	Poor transfection efficiency	See above section. Concentrate the virus using centrifugation (see Appendix A).
	Virus harvested too early	Harvest virus 48–72 hr after the start of transfection.
	Vector too large (The limit of packaging function is 9.7 kb from 5′-LTR to 3′-LTR)	Concentrate the virus (see Appendix A) for large vectors or reduce size of the insert.
	Polybrene missing or at suboptimal concentration	Add polybrene (4 µg/ml) during transduction or optimize the concentration (2–12 µg/ml)
	Virus exposed to multiple freeze-thaw cycles	Each cycle reduces titer by approximately 2–4 fold. Limit the number of freeze-thaws.
	Suboptimal selection procedure during titration	Perform an antibiotic kill curve on the cell line prior to using it for titration.

IX. Troubleshooting Guide continued

Table I. Troubleshooting Guide for Lenti-X Expression Systems (continued)		
D. Transduction of Target Cells		
1. Poor transduction efficiency	Low titer	See Section C.
	Transduction protocol not optimized	See Appendix B for references to help with optimizing transduction protocols.
	Low viability of target cells during transduction	Optimize culture conditions for target cells prior to infection
		Packaging cell line-conditioned media may affect cell growth; dilute viral supernatant or shorten exposure time to viral supernatant. Consider using RetroNectin® Reagent and the RetroNectin-Bound Virus transduction protocol.
		Excessive exposure to polybrene: optimize amount of polybrene (titrate) or shorten exposure time to viral supernatant
	Viral supernatant contains transduction inhibitors	Use RetroNectin Reagent or RetroNectin-coated plates in the RetroNectin-Bound Virus transduction protocol, which allows virions to bind the RetroNectin substratum and be washed free of inhibitors prior to target cell infection.
2. Low expression of GOI	Low transduction efficiency	See Section D.1.
	Promoter may be weak or possibly inactivated in target cells	Insert a tissue-specific promoter for GOI expression.
	Poor target cell viability	Check growth parameters.
3. Infection is toxic to target cells	MOI too high (i.e. too much virus used)	Dilute virus stock; titrate the virus.
	Polybrene toxicity	Reduce or optimize polybrene concentration; reduce infection time.
	Packaging cell supernatant or medium is toxic to cells	Dilute virus stock using target cell culture medium; harvest virus from packaging cells using target cell medium

X. References

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Appendix A: Additional Protocols

A. Protocol: Titrating Antibiotics for Selecting Stable Cell Lines.

Prior to using the antibiotics G418 (Cat. No. 631308) and/or puromycin (Cat. No. 631306) to select cells that have been either singly- or doubly-transduced with Lenti-X lentiviruses, it is necessary to titrate each selection agent to determine the optimal concentration for your target cell line. With HeLa cells, for example, we have found 400 µg/ml G418 and 1.0 µg/ml puromycin to be optimal.

Table II. Recommended Concentrations for Selection Antibiotics (µg/ml)

Antibiotic	Working Range	Selection	Maintenance
G418	50–800	400–500	100
Hygromycin	50–800	200	100
Puromycin	0.25–2	0.5–10	0.25

- For selecting stable transformants with G418 and hygromycin B, use the lowest concentration that results in massive cell death in ~5 days and kills all the cells within two weeks.
- Puromycin selection occurs more rapidly; use a concentration that will kill all cells within 3–4 days.
- Lot-to-lot variations in potency exist for all selection drugs, so each new lot of antibiotic should be titrated.

1. For each antibiotic to be tested, plate 2 x 10^5 cells in each well of a 6-well plate containing 3 ml of the appropriate complete medium plus increasing concentrations of G418 (0, 50, 100, 200, 400, and 800 µg/ml). For puromycin, add the drug at 0, 1.0, 2.5, 5.0, 7.5, and 10.0 µg/ml.
2. For G418, incubate the cells for 5–10 days or until all cells are dead. Examine the dishes for viable cells every two days. Replace the selective medium every four days (or more often if necessary), until the optimal concentration is determined.
3. For puromycin, incubate the cells 4–7 days. Replace medium after 2 days to remove dead cells.

B. Protocol: Concentrating Virus

This should be used for VSV-G-enveloped virions only (Burns *et al*, 1994).

1. Remove cell debris and aggregated virus by low speed centrifugation (500 x g) for 10 min at 4°C.
2. Pellet the virus at 50,000 x g for 90 min at 4°C. Remove the supernatant.
3. Resuspend the virus to 0.5–1% of the original volume in TNE (50 mM Tris-HCl [pH 7.8], 130 mM NaCl, 1 mM EDTA), and incubate overnight at 4°C.

   Note: If desired, perform a second round of ultracentrifugation (Steps 1–2).

4. Determine the viral titers of pre- and post-concentrated viral supernatants.
5. Transduce target cells.

Appendix B: Additional Viral Infection Methods

These references are provided for fine-tuning your transduction protocols so that you may improve your transduction efficiency in target cells. This list is not a comprehensive list, but many of these protocols will work for a wide range of cell types. You must determine which methods work best for your targets and certain methods may have additive effects. For optimization experiments, we recommend using one of our Lenti-X Fluorescent Vectors to express a Living Colors Fluorescent Protein, which simplifies the detection and quantitation of lentiviral gene transfer efficiency.

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B. Centrifugation during transduction ("spinoculation"), may counteract diffusion of virus when binding target cells

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G. Cocultivation of target cells and packaging cells: Allows targets to be continuously in contact with freshly-produced viral supernatant

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Germeraad, W. T., Asami, N., Fujimoto, S., Mazda, O. & Katsura, Y. (1994) Efficient retrovirus-mediated gene transduction into murine hematopoietic stem cells and long-lasting expression using a transwell coculture system. *Blood* **84**(3):780–788.

H. Use of cationic liposomes: Enhance virus-to-cell fusion

Kaneko, Y. & Tsukamoto, A. (1996) Cationic liposomes enhance retrovirus-mediated multinucleated cell formation and retroviral transduction. *Cancer Lett.* **105**(1):39–44.

Porter, C. D., Lukacs, K. V., Box, G., Takeuchi, Y. & Collins, M. K. (1998) Cationic liposomes enhance the rate of transduction by a recombinant retroviral vector in vitro and in vivo. *J. Virol.* **72**(6):4832–4840.

Appendix B: Additional Viral Infection Methods continued

I. Use of histone deacetylase inhibitors to increase titer: Relieves repression of viral expression by hyperacetylation of histones

Chen, W. Y., Bailey, E. C., McCune, S. L., Dong, J. Y. & Townes, T. M. (1997) Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase. *Proc. Natl. Acad. Sci. USA* **94**(11):5798–5803.

Tobias, C. A., Kim, D. & Fischer, I. (2000) Improved recombinant retroviral titers utilizing trichostatin A. *Biotechniques* **29**(4):884–890.

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Markova, S. V., Golz, S., Frank, L. A., Kalthof, B. & Vysotski, E. S. (2004) Cloning and expression of cDNA for a luciferase from the marine copepod *Metridia longa*. A novel secreted bioluminescent reporter enzyme. *J. Biol. Chem*. **279**(5):3212–3117.

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